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Exhibit 4

Analysis of the Individual Role of the TCRζ Chain in Transgenic Mice after Conditional Activation with Chemical Inducers of Dimerization

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Signaling through the TCR/CD3 complex plays a critical role in T-cell development and activation. Gene-targeted mice lacking particular components of this complex show arrested T-cell development in the thymus. As all TCR/CD3 components are required for efficient surface expression of the complex, it is difficult to assess the specific signaling role of each receptor component. To overcome this problem, we designed a strategy to examine the specific role(s) of individual receptor chains. A chimeric protein, containing binding domains for chemical inducers of dimerization fused to the cytoplasmic tail of TCRζ, was generated. Activation of the chimeric receptor after stimulation with chemical dimerizers in Jurkat cells showed tyrosine phosphorylation of the TCRζ chain chimera, recruitment of phosphorylated Zap70, and generation of NFAT in a reporter assay. Analysis of thymocytes from transgenic mice expressing this chimeric receptor showed that intracytoplasmic cross-linking of the chimera induced tyrosine phosphorylation of the protein, as well as a slow and very weak calcium mobilization response. However, this signaling did not lead to increased expression of activation markers, T-cell proliferation, or apoptosis. In addition, stimulation of thymocytes in suspension or in fetal thymic organ cultures with chemical inducers of dimerization did not lead to alterations in positive or negative selection. We conclude that signaling through the TCRζ chain alone is not sufficient to generate downstream events leading to full T-cell activation or thymocyte selection; instead, additional CD3 components must be required to induce a functional response in primary thymocytes and peripheral T cells. © 2001 Elsevier Science (USA)

Key Words: **T lymphocyte; thymocyte; signal transduction; cellular differentiation; TCRζ chain; TCR/CD3 complex; T cell receptors; dimerizer; ITAM; transgenic mice.**

INTRODUCTION

The T-cell receptor (TCR) complex on mature thymocytes and T lymphocytes is composed of the α- and β-chains, which determine the MHC/peptide specificity of the receptor, and four noncovalently associated polypeptides, CD3ε, CD3δ, and CD3γ and a dimer of TCRζ. The CD3 chains plus TCRζ constitute the signaling components of this complex and function by recruiting tyrosine kinases to their cytoplasmic tails after TCR engagement. This recruitment is mediated by the presence of one ITAM (immunoreceptor tyrosine-based activation motif) consensus sequence in the tail of each CD3 subunit plus three ITAM sequences in the TCRζ tail (1). The ITAMs consist of pairs of YXXL/I motifs separated by six to eight variable amino acids. Phosphorylation of the two tyrosines in each ITAM by an src-family kinase (Lck or Fyn) allows the recruitment of the tandem SH2 domain-containing cytoplasmic tyrosine kinase Zap70 (or Syk). After recruitment to the TCR, Zap70 is phosphorylated and induces recruitment and activation of other effector and adapter molecules (2). This leads to a phosphorylation cascade that ultimately results in the activation of the calcium and MAP kinase signaling pathways (3) and the generation of other downstream effectors, culminating in the activation and differentiation of the T lymphocyte (reviewed in 4).

TCR signaling plays a critical role at several stages of T-cell development in the thymus. The ultimate outcome of this process is the maturation of a self-MHC-restricted, self-tolerant repertoire of mature CD4+ and CD8+ T cells (5). The stages of T-cell development can be identified by examining the surface expression of CD4, CD8, TCR/CD3 molecules on thymocytes. The earliest thymocyte subset, $CD4^-CD8^-CD3^-$, triple-negative (TN) cells, can be further subdivided into four stages based on expression of CD44 and CD25 (TN1–TN4) (6). During T-cell development two different forms of the antigen receptor are expressed at distinct developmental stages. At the TN3 stage, thymocytes

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express the pre-TCR, composed of the mature TCRβ chain paired with the pre-Tα chain. This pre-TCR is also associated with the invariant CD3-ε and -γ chains and the TCRζ chain. The signal transduced by the pre-TCR mediates "β selection," a process that promotes the transition of thymocytes from TN3 to TN4 and then to $CD4^+CD8^+$, double-positive (DP) cells. Pre-TCR signaling also induces TCRα chain rearrangement (7). Double-positive thymocytes then express a mature αβ-TCR and undergo positive and negative selection (8). Positive selection promotes the maturation of thymocytes whose TCRs are able to recognize peptide–MHC complexes on cortical epithelial cells with low avidity, while negative selection is responsible for the elimination of thymocytes whose TCRs have a very high avidity for MHC–peptide complexes in the thymus.

Among the TCR-associated chains, TCRζ is particularly interesting due to the presence of three ITAMs in its cytoplasmic tail, allowing for multiple phosphorylation states (9). This multiplicity of ITAMs in the TCRζ chain has been postulated to have a quantitative and/or qualitative role in TCR signal transduction and is important for the regulation of TCR-mediated signals during both T-cell development and T-cell activation (10). In TCRζ/η-deficient mice, differentiation of thymocytes is blocked at the DP stage (11), suggesting that TCRζ is required for αβ-TCR repertoire selection. However, this phenotype is due to the inability of the mature αβ-TCR to be assembled and expressed on the cell surface in the absence of TCRζ. Specifically, transgenic mice containing ITAM-deficient TCRζ chains were able to restore T-cell development in ζ/η-deficient mice (12, 13). Therefore, in the absence of TCRζ ITAMs, other CD3 ITAMs can transduce sufficient TCR signals to mediate positive selection. This is also the case for β selection, as TCRζ, in contrast to the CD3ε chain, is dispensable for this process (14), although recent experiments indicate that ζ-chain dimers may contribute to pre-TCR signaling (15).

Among the *in vivo* experiments supporting the quantitative role of TCRζ in TCR signal transduction are those in which transgenic TCRζ chains with different number of ITAMs were used to reconstitute ζ-deficient mice. One such study showed that all TCRζ ITAMs are required for the normal development of thymocytes expressing the H-Y TCR (13). Furthermore, studies using a transgenic TCR presumed to have a higher affinity for its ligand (i.e., P14) demonstrated that thymocytes in these mice could be selected in the absence of some ITAM sequences and, further, that activation of mature P14+ T cells was also relatively insensitive to the absence of some TCRζ ITAMs (16).

Additional studies have also supported the idea that the TCRζ ITAMs do not have a unique and nonredundant role in T-cell development or activation. For instance, analysis of transgenic mice expressing either TCRζ or CD3ε cytoplasmic tails fused to the extracellular domain of the human IL-2 receptor α-chain (Tac) showed that activation through *in vivo* injection of the anti-Tac antibody could restore differentiation of DP thymocytes in Rag-2 KO mice (17). These data led the authors to conclude that TCRζ and CD3ε can deliver similar signals during T-cell development. However, additional experiments assessing the role of the TCRζ chain ITAMs independent of the other CD3 subunits have provided controversial results. Early reports using cell lines demonstrated that the TCRζ cytoplasmic tail alone could generate T-cell activation signals (1, 18), a finding that was reproduced in one transgenic mouse model (19). Conversely, other studies using TCRζ chain chimeras failed to generate T-cell activation signals after signaling through the ζ-chain cytoplasmic tail alone (20).

In contrast to these genetic systems, biochemical studies support the idea that differential phosphorylation of the TCRζ ITAMs may serve to deliver qualitatively different signals resulting in distinct functional responses. For instance, synthetic phosphopeptides containing individual ITAMs show preferential binding to distinct adapter molecules (e.g., Shc, Grb-2, or the p85 subunit of PI3K) (21). In addition, chimeric constructs containing the cytoplasmic tails of either TCRζ or CD3ε induced distinct calcium mobilization (22) and activation-induced cell death responses (23). Despite these *in vitro* findings, there is as yet no *in vivo* evidence to support ITAM specificity in transgenic mice expressing TCRζ chains containing different ITAM sequences (reviewed in 10).

Another component regulating TCRζ signaling is the sequential phosphorylation of individual ITAMs following TCR engagement. Based on these data, Allen and colleagues proposed a model for how the TCR translates quantitative differences in antigen binding into qualitatively different biological responses (24). These investigators also demonstrated that all ITAMs need to be phosphorylated to fully activate mature T lymphocytes and, further, that partial phosphorylation of the TCRζ chain could give negative signals and prevent T-cell activation (25). These data have provided a potential mechanism to account for the different responses obtained after stimulation of cells with agonist, partial agonist, or antagonist peptides and indicate that different phosphorylation states of TCRζ may play a critical role in translating ligand binding signals into distinct functional responses. In a recent report van Oers and collaborators (26) showed that the p21 and p23 phosphorylation states of the TCRζ chain are composed by phosphorylation of specific tyrosine residues, which differ from those reported by Allen and coworkers. However, both groups agree on the existence of a hierarchy of TCRζ ITAM tyrosine phosphorylation during T-cell activation and on the fact that p23 corresponds to the fully phosphorylated TCRζ chain.

Given the complexity of the TCR/CD3 complex, and the TCRζ chain in particular, it is perhaps not surpris-

ing that different experimental systems have produced conflicting results. One issue concerning many of the *in vitro* and *in vivo* systems is that signaling through chimeric receptors was often achieved by extracellular crosslinking using specific monoclonal antibodies. This method induces massive activation of virtually all surface receptor molecules, a situation that is not likely to be comparable to the natural physiological interactions of TCR complexes with MHC/peptide ligands on an APC. To overcome this problem, we used a system capable of intracellular receptor aggregation in which the level of crosslinking can be controlled by altering the dose of the dimerizing drug (CID, chemical inducer of dimerization). Initially, this system was shown to induce signaling via the TCRζ cytoplasmic tail using the FK1012 dimerizer in transfected tumor T-cell lines (27). Newer versions of dimerizers, such as AP1510, are very potent at inducing receptor dimerization and transcriptional activation in cells expressing appropriate FKBP fusion proteins (28) and has also been successfully used in studies of signaling pathways involving src-like tyrosine kinases, Grb2 and Sos adapter molecules (29), the PDGF receptor (30), Fas (31), Bax (and caspases) (32), c-kit (33), and $\alpha_{IIb}\beta_3$ integrins (34). Furthermore, this method has also been tested *in vivo*, allowing the cell-type-specific activation of a cell death signaling pathway (35) and the regulated production of human growth hormone in mice (36).

In this study we have applied this approach to the conditional activation of the TCRζ chain *in vitro* and *in vivo*. We generated a chimeric construct consisting of the extracellular and transmembrane domains of the human CD2 molecule; the cytoplasmic tail of murine TCRζ; followed by three copies of the immunophilin FKBP-12; and last, a peptide from the influenza virus hemagglutinin protein (HA-Tag). We have assessed signaling through this chimeric protein in Jurkat T-cell transfectants and in transgenic mice expressing the chimeric receptor under control of the human CD2 promoter. We demonstrate that intracellular crosslinking of the cytoplasmic TCRζ chain can induce signals sufficient for IL-2 production in Jurkat T-cell lines, but is unable to induce a functional response from primary T lymphocytes or thymocytes.

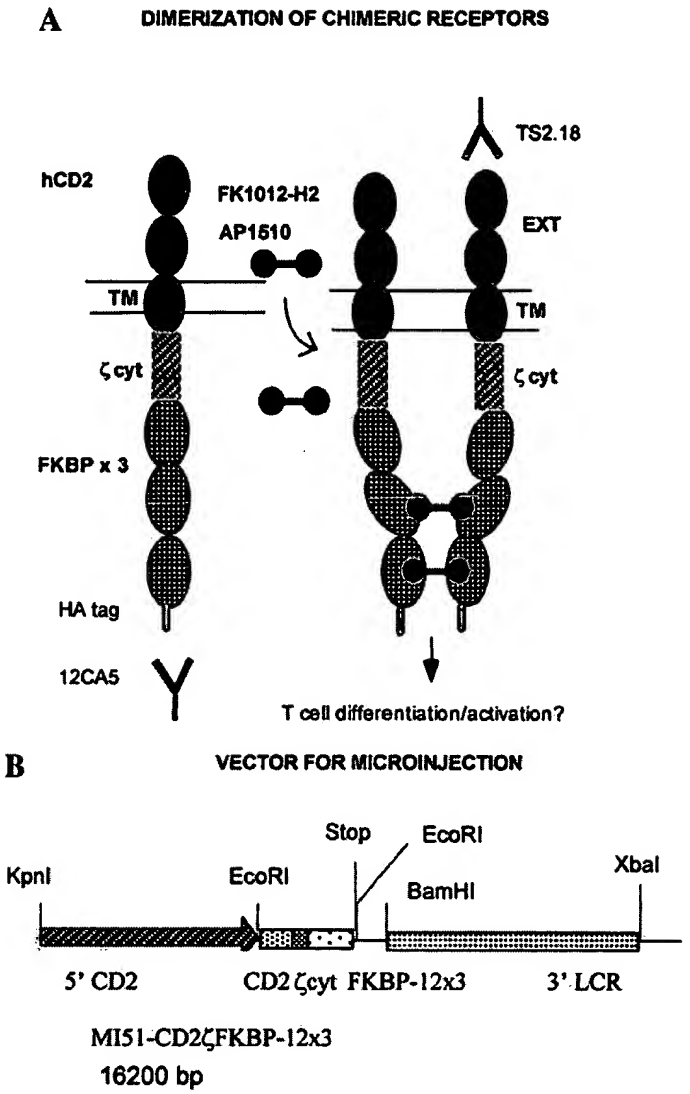

FIG. 1. (A) Dimerization system used in this study. Addition of the membrane permeable dimerizer, FK1012-H2 or AP1510, induces intracellular crosslinking of the chimeric receptor upon binding to its specific ligand, FKBP-12. (B) Microinjection vector used for the generation transgenic mice containing a 5.5-kb 5′ CD2 promoter sequence and the 5-kb 3′ locus control region sequence, which allows T-cell-lineage-specific expression of the transgenic protein. The 13-kb *Kpn*I–*Xba*I fragment was used for the microinjection.

MATERIALS AND METHODS

Generation of Chimeric Constructs

For the CD2-ζ-FKBP-12×3 chimeric construct, a ~700-bp fragment containing the extracellular and transmembrane domains of the human CD2 molecule was PCR amplified using Pfu polymerase (Stratagene, La Jolla, CA) and cloned into the Bluescript (pSK-) polylinker sequence as a *Xba*I–*Xho*I fragment. The 320-bp cytoplasmic tail of murine TCRζ chain was isolated from the vector MZE (27) and was ligated to the 3′ end of the hCD2 sequence at the *Xho*I site. Three copies of the human FKBP-12 (~1 kb) containing the mutations G89P and 190K (37) were excised (*Xho*I–*Eco*RI) from the M3FE construct and ligated in frame downstream of the CD2-ζ. For expression in eukaryotic cells the *Not*I–*Eco*RI hCD2-ζ-FKBP fragment was subcloned into a derivative of the PBJ5 vector (27). For the generation of transgenic mice, this chimeric construct was introduced into the *Eco*RI site of the MI51 vector (kindly provided by Dr. Dimitri Kioussis, Mill Hill, London, UK), containing the 5 kb 5′ hCD2 promoter sequence and the 5.5-kb 3′ CD2 locus control region (LCR) (38 and Fig. 1B).

Another chimeric construct containing the same extracellular and transmembrane domains fused to the

cytoplasmic tail of murine CD4 and followed by the FKBP-12 ×3 sequence (CD2-CD4-FKBP-12 ×3) was generated and used in cotransfection experiments. The originally described TCRζ chimeric receptor, MZF3E (27), containing a myristoylation sequence instead of the hCD2 extracellular and transmembrane domains (myr-ζ-FKBP-12 ×3), was also used in transient transfections of Jurkat Tag cells.

Chemical Inducers of Dimerization

A modified version of the original FK1012-A dimerizer, FK1012-H2, was synthesized by Dr. Steve Diver at the Chemical Biology Department at Harvard University using a one-step synthesis protocol that allows the generation of CIDs with a shorter linker than the originally described FK1012 (39). AP1510 is a fully synthetic dimerizer of a smaller size and more potent activity than FK1012-H2 (28) and was kindly provided by Ariad Pharmaceuticals (Cambridge, MA).

Expression and Function of the Chimeric Receptor in Jurkat T Cells

Five micrograms of DNA were transiently transfected into 10^7 Jurkat Tag cells using a BTX electroporator (Model No. 600; Genetronics Inc., San Diego, CA), 960 μF, 129 Ω, 250 Volts, in 200 μl RPMI- 1640 medium (Gibco/BRL, Carlsbad, CA). Cells were plated in 10% FCS/RPMI medium and 24 h later the medium was changed and replaced with 0% FCS/RPMI for another 18–24 h. Surface staining of transfected cell lines was performed using TS2.18 antibody (provided by Dr. Barbara Bierer, Dana Farber Cancer Institute, Boston, MA)(40), specific for the hCD2 extracellular domain of the TCRζ chimera. Anti-influenza virus hemagglutinin (HA) antibody (12CA5, hybridoma from ATCC) was used to immunoprecipitate total lysates from transiently transfected Jurkat Tag cells. Forty-eight hours after transfection, cells were harvested and stimulated with CID (0.1–1 μM) for 1 to 30 min. As control, cells were stimulated for 3 min with anti-CD3 (OKT3, hybridoma from ATCC) or hCD2-biotin (TS2.18) followed by crosslinking with a secondary reagent, rabbit antimouse Ig or streptavidin (both from Sigma, St. Louis, MO). Preparation of total cell lysates and immunoprecipitations were performed as described (41). Immunoprecipitates were resolved on 10% SDS–PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Western blots were performed with anti-HA tag antibody (12CA5) and antiphosphotyrosine antibody, 4G10 (kindly provided by Dr. B. Drucker, Oregon Health Sciences Center, Portland OR). To analyze downstream signaling events, lysates were immunoprecipitated with anti-Zap70 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and Western blot analysis was performed with 4G10. For immunoblots, peroxidase-labeled antimouse or antirabbit Ig antibodies (Amersham Pharmacia Biotech Inc., Piscataway, NJ) were used as secondary reagents, and immunoreactive bands were detected by ECL (Amersham Pharmacia Biotech Inc.).

Secreted Alkaline Phosphatase Assay (SEAP)

Jurkat-Tag cells (10^7) were transiently transfected with 5 μg of the hCD2-ζ–FKBP12 ×3 chimeric construct, plated in 96-well plates at 7×10^5 cells per well in 10% FCS/RPMI, and stimulated overnight with either phorbol myristate acetate (PMA, 2 nM) plus ionomycin (1 μM) or FK1012-H2 (0.1–1 μM). Supernatants were assayed, in triplicate, for SEAP activity by the method of Spencer *et al.* (27). A myristoylated chimeric construct (MZF3E) (27) and a CD2–CD4–FKBP-12 ×3 chimera (see above) were used in parallel experiments as positive and negative controls, respectively, for IL-2 production in the SEAP assay.

Generation of Transgenic Mice

Transgenic mice were generated using the CD2-ζ–FKBP-12 ×3 insert in the MI51 vector containing the human CD2 minigene. This vector has been successfully used in the generation of transgenic mice with restricted expression of the transgene to the T-cell lineage (38). For microinjection, the vector was digested to remove the bacterial vector sequences and the remaining ~13-kb fragment was gel purified and microinjected into fertilized B6/CBAF1-J oocytes by standard procedures. Injected embryos were transferred into pseudopregnant B6/CBAF1-J female mice, and pups were screened for carrying the transgene construct by Southern blot analysis. Individual founder mice were backcrossed to B10.BR mice, and progeny were examined for expression of the chimeric protein by flow cytometry analysis using an anti-hCD2 mAb (TS2.18). Of the six founders, five had detectable surface expression of the chimeric protein. Experiments were all performed with line 26, which had the highest detectable surface expression of the chimera. Once transgenic line 26 was established, mice were screened by PCR using hCD2 specific primers as follows: CD2-5′ (5′-CCTAAGATGAGCTTTCCATGT-3′) and CD2-3′ (5′-CAGTTCCATTCATTACCTCAC-3′). For the Rag-1 and ζ/η-knockout (KO) mice, Neo^R sequences were identified using the following primers: Neo-5′ (5′-ATTGAACAAGATGGATTGCAC-3′) and Neo-3′ (5′-CGTCCAGATCATCCTGATCC-3′). Detection of the intact Rag-1 allele was performed with the following primers: Rag-1, -5′ (5′-CAGCTATTGTCCCTCTTGCCGATATCCGTG-3′); and Rag-1: -3′ (5′-ATGGTGGTTATATTTTTCCAGACTCCTTCA-3′). Detection of the intact TCRζ chain allele was performed with primers to exon 2 as follows: zeta-5′ (5′-AGGCACAGAGCTTTGGTCTGC-3′) and zeta-3′ (5′-TTTTGCTCTCAGGTACAGGGC-3′). ζ/η-KO mice were obtained from the CNRS-INSERM (Marseille, France) (11). Rag-1 KO mice were previously described (42). These mice were maintained in

SPF conditions at the University of Massachusetts Medical School vivarium and in the mouse facility at the Instituto de Investigaciones Biomédicas (UNAM, México D.F., México). Transgenic founder mice and KO mice were backcrossed to B10.BR mice for more than four generations.

Flow Cytometry Analysis

Lymph node cells and thymocytes from transgenic mice and nontransgenic littermates were stained with a triple-antibody cocktail composed of the following: antihuman CD2 (TS2.18) biotin (kindly provided by Dr. Barbara Bierer), antimouse CD4-cychrome, (PharMingen, San Diego, CA), and antimouse CD8-FITC (PharMingen) followed by streptavidin-PE (Southern Biotechnology Associates, Birmingham, AL, and PharMingen). Cells were analyzed on a FACsCalibur flow cytometer (Becton–Dickinson, Mountain View, CA) and data were analyzed using CellQuest software. Flow cytometer was previously calibrated using the Calibrite 3 reagent (Becton–Dickinson) and the software program FACs Comp (Becton–Dickinson). Lymphocyte populations were defined using a on a Forward/Side Scatter gate. Prior to staining, Fc receptors were blocked using normal mouse serum at a concentration of 1/20.

Protein Biochemistry Analysis from Transgenic Mice

Thymocytes and lymph node cells were isolated and incubated for 2 h in RPMI/0.5% FCS at 37°C prior to stimulation. Cells were then washed once with RPMI/0% FCS and 10×10^6 cells were resuspended in 300 μl of either RPMI/0% FCS or this medium containing CID (0.1–1 μM). Stimulations were terminated and total lysates prepared as described above. As a positive control, cells were incubated with anti-CD3-biotin (5 μg/ml) (PharMingen) plus anti-CD4 biotin (5 μg/ml) (PharMingen) for 10 min at 4°C followed by crosslinking with streptavidin (10 μg/ml) (Sigma) for 3 min at 37°C. Immunoprecipitations and Western blots were performed as described above. Anti-TCRζ and anti-Zap70 antibodies (Santa Cruz Biotechnology) were used to immunoprecipitate the TCRζ chimeric receptor from transgenic T cells.

Activation Assays

Lymph node cells (2.5×10^5), thymocytes (2.5×10^5), or splenocytes (10^5) isolated from transgenic mice (line 26) or nontransgenic littermates were used as responder cells in proliferation assays. Irradiated splenocytes (3000 rads) from MHC-matched littermates were added as APCs (5×10^4) to lymph node cell and thymocyte assays. Cultures were stimulated with CID (FK1012-H2 or AP1510 1 μM) for 3 days or with anti-CD2-biotin (TS2.18, 5 μg/ml) followed by streptavidin (10 μg/ml). As a positive control, cells were plated in wells precoated with goat antihamster Ig(H+L) (5 μg/ml) (Boehringer Mannheim, Mannheim, Germany) followed by hamster antimouse CD3ε (145-2C11, PharMingen) (5 μg/ml) and anti-CD28 (1:8 dilution of antibody supernatant 37N.D1; a gift from Dr. J. Allison) (43). Parallel cultures were stimulated with PMA (1 ng/ml) and ionomycin (500 ng/ml). As a negative control, responder cells were cultured alone or in the presence of irradiated APCs. All stimulations were performed in triplicate. After 3 days of culture 1 μCi of [^{3}H]thymidine was added per well, and cells were harvested 18–22 h later and incorporation was measured in a beta counter. For prestimulation assays, total lymph nodes were incubated for 48 h on anti-CD3-(2C11, PharMingen) coated wells (5 μg/ml) and incubated for 24 or more h in the presence of recombinant hIL-2 (50 U/ml, Boehringer Mannheim). Cells were then stimulated with CID or antibody crosslinking, as previously described. For analysis of activation markers, thymocytes or lymph node cells were plated at 10^5 cells/well in 96-well plates and incubated for 24 h in the presence or absence of CIDs. Control stimulations were as described above.

Calcium Mobilization

Freshly isolated lymph node cells (5×10^6) or thymocytes were labeled using Fluo-3 and Fura-Red dyes (Molecular Probes, Eugene, OR) as described (44). Stimulation was performed by adding CIDs (1 μM) or anti-CD3-biotin (5 μg/ml) plus anti-CD4-biotin (5 μg/ml) followed by streptavidin (10 μg/ml). Ionomycin (500 ng/ml) was added at the end of the stimulation as a control for calcium dye loading. Stimulated cells were analyzed on a FACsCalibur Flow Cytometer, using CellQuest software (Becton–Dickinson). Calcium flux was calculated and represented as the ratios FL1/FL3 using the computer program FACs Assistant (Becton–Dickinson).

Fetal Thymic Organ Cultures (FTOC)

FTOC of day 16 and day 17 fetal thymi were performed as described (45). Cultures were stimulated with complete media or media supplemented with dimerizer (0.1–1 μM). Six days later cells were stained with anti-CD4-cychrome, anti-CD8-FITC (PharMingen), and anti-hCD2-biotin followed by streptavidin-PE (Southern Biotechnology Associates and PharMingen).

Dulling Assay

Thymocytes from 6- to 10-week-old transgenic mice or control littermates were isolated and plated at 10^5 cells/well in 96-well plates. Cells were incubated in medium alone or in medium containing 0.1 or 1 μM CIDs (AP1510 or FK1012-H2). Parallel cultures were incubated in anti-CD3 or anti-CD3+ anti-CD28 precoated wells as a positive control for this dulling assay.

Analysis of coreceptor downregulation was performed as described (46).

RESULTS

Generation of the Chimeric Signaling Protein

To generate a TCRζ chain receptor capable of signaling independently from the TCR/CD3 complex, we created a chimeric protein (Fig. 1A). This protein carries the cytoplasmic tail of the murine TCRζ chain and three copies of the immunophilin, FKBP-12, followed by an HA tag. FKBP-12 is the cellular ligand for the immunosuppressive drug FK506 and also binds the synthetic chemical inducer of dimerization (CID), FK1012. The strategy used in this study is based on the ability of CIDs to induce intracytoplasmic crosslinking of receptors that contain CID-binding sites. Thus, addition of the CID into the extracellular media will induce conditional activation of the TCRζ chain (Fig. 1A). Three copies of the FKBP-12 domain were included, as previous reports indicate that TCRζ chimeric constructs carrying three FKBP-12 domains allow oligomerization rather than simple dimerization of the chimeric receptor and give a stronger CID-dependent activation signal in T-cell transfectants (27). To allow for cell surface targeting and detection of the chimeric receptor, the TCRζ/FKBP-12 cytoplasmic domain was fused to the extracellular and transmembrane domains of the human CD2 protein. In addition, to avoid the complications of CD2 binding activity, we used a point mutant of human CD2, carrying a substitution (Q46L) in the ligand-binding domain. This mutation ensures lack of binding of hCD2 to human CD58 as well as to CD48, the murine receptor for CD2 (40).

Expression and Function of the Chimeric Receptor in T-Cell Lines

Prior to the generation of transgenic mice the chimeric receptor was tested *in vitro* in transfected Jurkat T cells. Cells were stimulated with the CIDs and tyrosine phosphorylation analysis was performed. These experiments demonstrated that both FK1012-H2 and AP1510 induced tyrosine phosphorylation of the TCRζ chimera as early as 1 min after stimulation, with maximal phosphorylation at 20–30 min (Fig. 2A). A myristoylated chimeric construct, containing the same TCRζ cytoplasmic tail and three copies of FKBP-12 (myr-ζ) was also used which had previously been shown to activate Jurkat Tag cells after FK1012-H2 (27). As a control, cells were stimulated with biotinylated anti-hCD2 antibody (TS2.18) followed by streptavidin crosslinking, resulting in a very strong tyrosine phosphorylation of the chimeric receptor (not shown). Immunoprecipitation of the chimeric receptor was detected by immunoblotting with anti-HA antibody (12CA5) (Fig. 2B), and surface expression of the chimera was confirmed by flow cytometry (data not shown). Furthermore, we used anti-Zap70 antibody to assess whether the TCRζ chain chimera was able to recruit phosphorylated Zap70. As shown in Fig. 2C, phosphorylated CD2ζFKBP-12 × 3 chimera was coimmunoprecipitated with Zap70, indicating that phosphorylation of this chimeric protein after CID stimulation led to the recruitment and activation of downstream signaling molecules. Recruitment of phosphorylated Zap70 was clearly increased when a CD4 chimeric construct was cotransfected with the TCRζ construct (Fig. 2C), indicating that recruitment of the CD4-associated signaling molecules (such as Lck) contributes to the signaling ability of the TCRζ chimeric receptor. While the initial experiments were performed using the FK1012-H2 dimerizer, later experiments used a synthetic CID, AP1510, which was shown to be significantly more efficient than FK1012-H2 at inducing phosphorylation of the TCRζ chimera (not shown).

In order to assess the ability of the TCRζ chimera to generate signals for T-cell activation we used a reporter assay as an indirect measurement of IL-2 gene induction. Jurkat Tag cells were cotransfected with the TCRζ chimera plus a reporter construct containing NFAT binding domains driving the expression of the secreted alkaline phosphatase (SEAP) gene. Activation of the TCRζ chimera with 0.1–1 μM FK1012-H2 for 24 h induced SEAP secretion into the extracellular medium (Fig. 3). Another TCRζ chimeric construct, myr-ζ-FKBP ×3, (MZ3FE) (27), which contains a myristoylation signal in place of the hCD2 extracellular and transmembrane domains, also induced NFAT activation in response to 0.1 and 1 μM concentrations of FK1012-H2. In contrast no SEAP was detected after stimulation of cells expressing a chimeric construct that contains the cytoplasmic tail of the murine CD4 molecule in place of TCRζ, confirming the fact that NFAT transcription factors are not activated in response to signaling through CD4 alone. In conclusion, these data demonstrate that intracellular crosslinking of the CD2ζFKBP-12 ×3 chimeric receptor is able to induce functional activation of Jurkat T cells.

Generation of Transgenic Mice Expressing the Chimeric TCRζ Chain Receptor

To test the ability of signaling through TCRζ alone to induce physiologically relevant signaling in primary thymocytes and T cells, we generated transgenic mice expressing the chimeric receptor. The chimeric construct was inserted into the CD2 expression vector, MI51, which contains the 5′ promoter and the 3′ locus control region of the human CD2 gene (Fig. 1B). When introduced into transgenic mice, this vector leads to T-cell-specific, integration-site-independent expression of linked genes (38). After injection of this construct into fertilized mouse eggs, 6 (of 29) independent transgenic founder lines were identified by Southern blot analysis (not shown). These founder mice were ana-

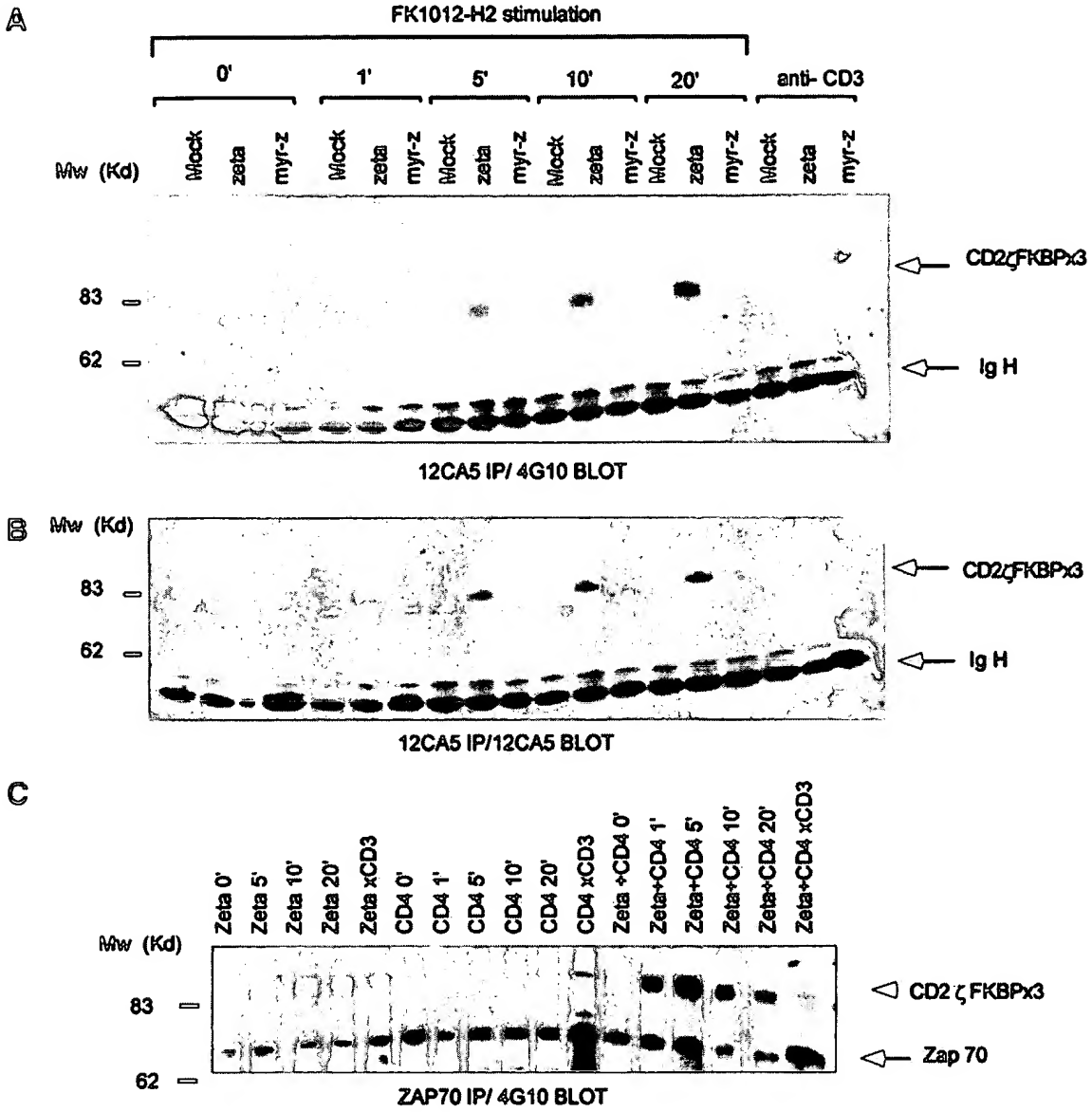

FIG. 2. Activation of Jurkat Tag cells after crosslinking of the CD2ζFKBP-12 ×3 chimeric protein with CID. Time-course experiment of transiently transfected Jurkat Tag cells stimulated with FK1012-H2 for 0–20 min. Three different constructs were used in this assay: (1) CD2-TCRζcyt-FKBP-12 ×3, (2) CD2-CD4cyt-FKBP-12 ×3, and (3) myristoylated TCRζcyt-FKBP-12 ×3. As controls, Jurkat Tag cells were electroporated in the same media without plasmid DNA (Mock). (A) Western blot analysis (4G10) of lysates inmunoprecipitated with anti-HA tag (12CA5) from cells stimulated at different times with dimerizer. As shown, an ~85-kDa TCRζ chimera (1) becomes tyrosine phosphorylated as early as 1–5 min after stimulation. The myristoylated chimera (2) also becomes tyrosine phosphorylated but it is not visible in the blot due to its molecular weight, which overlaps with the IgH band. Note that anti-CD3 crosslinking induces no phosphorylation of the chimeric TCRζ protein. (B). The same blot was stripped and probed with 12CA5 antibody to assess the expression of the chimeric protein. (C). Recruitment of Zap70 after activation of the TCRζ chimera. Transfections were performed using the CD2-TCRζcyt-FKBP-12 ×3 (zeta) or the CD2-CD4cyt-FKBP-12 (CD4) chimeric contructs alone or in combination. Immunoprecipitation of phosphorylated Zap70 was performed of total lysates obtained from transiently transfected Jurkat Tag after stimulation with CID (time 0 to 20, in minutes) or anti-CD3 crosslinking. As shown, tyrosine phosphorylated TCRζ chimera coimmunoprecipitated with Zap70, indicating that CID-induced activation of the TCRζ chimera (zeta) induces recruitment of phosphorylated Zap70. The level of TCRζ chimera coimmunoprecipitated was increased when cotransfected with CD2-CD4cyt-FKBP-12 (CD4), possibly indicating an effect of the recruitment of CD4 associated Lck to the signaling complex.

lyzed to determine the cell surface expression of the chimeric receptor by FACS analysis of peripheral blood lymphocytes using an antibody directed to the extracellular domain of human CD2 (hCD2). Line 26 was chosen for further study, as T cells from mice of this line had the highest levels of expression of the chimeric receptor. As shown in Fig. 4, all subsets of thymocytes express the chimeric receptor (Fig. 4A), as do peripheral T cells (Fig. 4B) from transgenic mice but not from nontransgenic littermates (bottom panels). In addition, expression of the chimeric receptor on thymocytes and peripheral T cells did not alter the numbers (data not

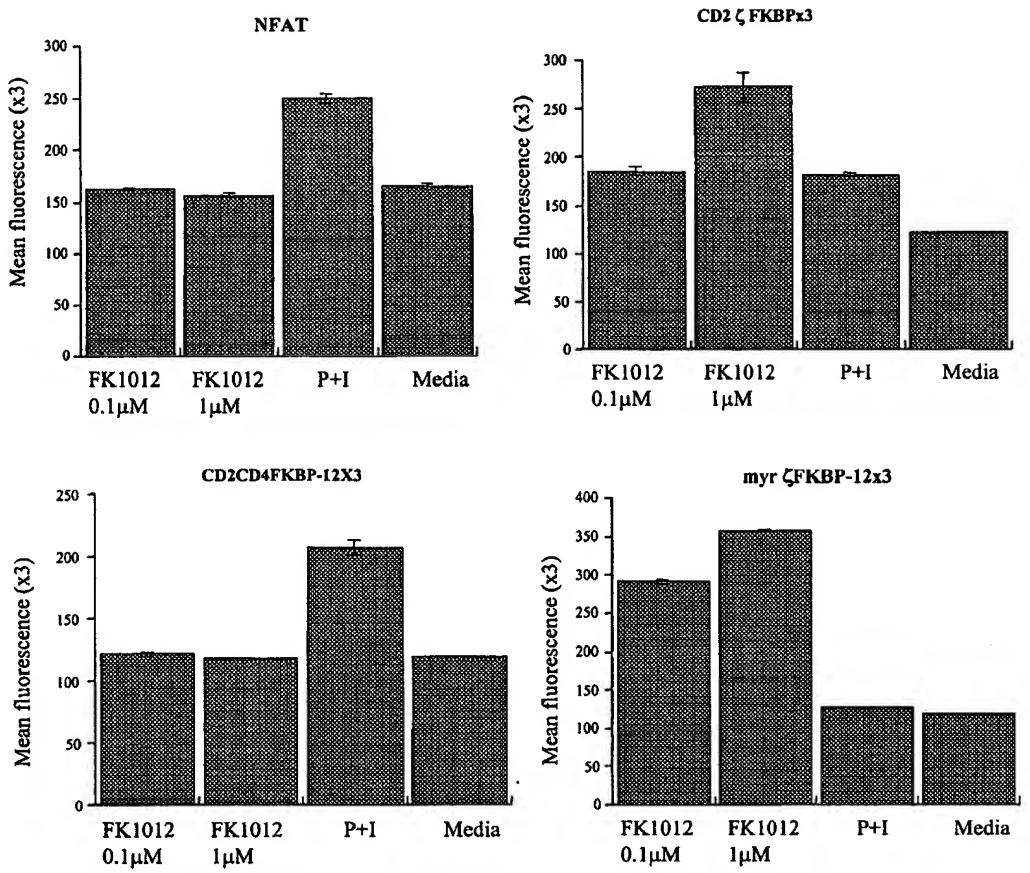

FIG. 3. Secreted alkaline phosphatase (SEAP) assay. Jurkat Tag cells were transfected with CD2ζFKBP-12 ×3, CD2-CD4-FKBP-12 ×3, MZ3FE (myristoylated TCRζ chimera) and the reporter construct NFAT (26) or with the NFAT construct alone (Control). Supernatants were assayed for SEAP 48 h after transfection to assess generation of NFAT transcription factors, which drive the expression of alkaline phosphatase. Values represent mean fluorescence (from triplicate cultures) in OD units at 460 nm. Significant SEAP was detected in Jurkat Tag cells transfected with the CD2ζFKBP-12 ×3 after stimulation with 0.1 and 1 μM FK1012-H2 (as well as in myristoylated form of this chimera myr-ζ-FKBP-12 ×3), but not in cells transfected with CD2-CD4-FKBP-12 ×3. Controls transfected with the NFAT reporter vector alone showed significant SEAP after treatment with PMA+ionomycin but not in response to FK1012-H2 treatment.

shown) or percentages of thymocyte or peripheral T cell subsets (Figs. 4A and B).

Crosslinking of the TCRζ Chimeric Receptor Induces Receptor Tyrosine Phosphorylation

To determine whether the TCRζ chimeric receptor was competent to transduce intracellular signals, we examined whether crosslinking of the receptor could induce receptor tyrosine phosphorylation. For these experiments, total thymocytes or lymph node cells from transgenic or nontransgenic mice were stimulated with the CID AP1510. AP1510 is a fully synthetic dimerizer previously reported to induce activation of signaling molecules in a number of studies (31–35). Cells were incubated at 37°C for 1 to 30 min in media containing 1 μM FK1012-H2 or AP1510. As a control, chimeric receptors were crosslinked by treatment with anti-hCD2 antibody. After stimulation, thymocytes and lymph node cells were lysed and the chimeric protein was immunoprecipitated with an antibody to the TCRζ chain or, alternatively, with an antibody directed against the HA tag. Immunoprecipitates and total cell lysates were fractionated and analyzed by immunoblotting with an antiphosphotyrosine antibody.

As it is shown in Fig. 5, a phosphorylated protein migrating at ~85 kDa, the expected size of the chimeric receptor, is clearly detected in immunoprecipitates from stimulated transgenic thymocytes. A similar result was obtained after stimulation of transgenic lymph node cells (not shown), but not with cells obtained from nontransgenic littermates (Fig. 5 and data not shown). Tyrosine phosphorylation of the chimeric protein was observed as early as 1–5 min after stimulation with the CID and increased up to 20 min after stimulation. In contrast, no phosphorylation of the chimeric receptor was observed following anti-CD3 plus anti-CD4 antibody crosslinking, indicating that the chimeric receptor is not associating with the native TCR/CD3 complex. Likewise, treatment with CID did not induce phosphorylation of the endogenous TCRζ chain (data not shown), which further supports the lack of association of the chimeric receptor with the

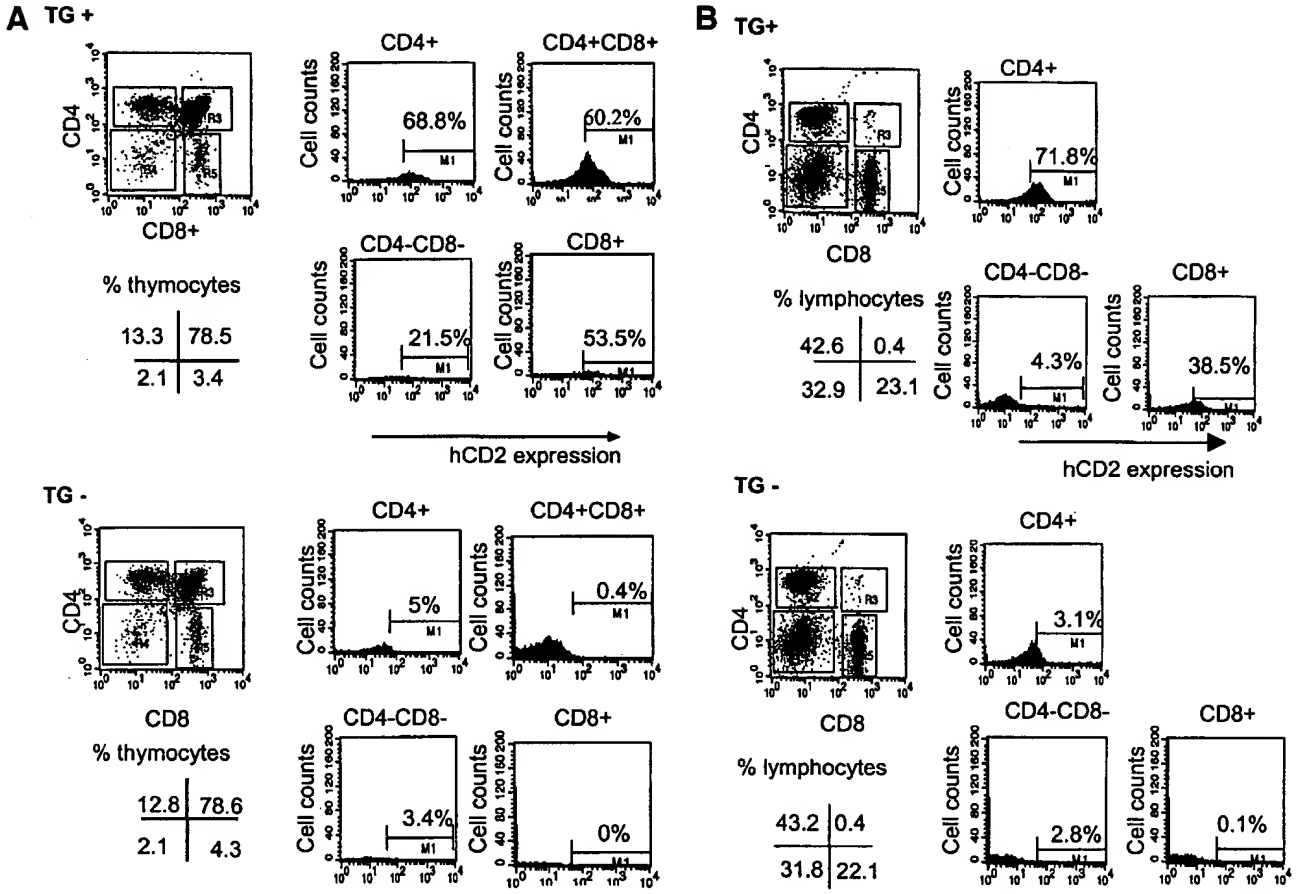

FIG. 4. Flow cytometric analysis of the chimeric receptor in transgenic mice. Total thymocytes (A) or lymph node cells (B) from a 4-week-old transgenic mouse (line 26) were triple stained with anti-CD4-cychrome, anti-CD8-FITC, and anti-hCD2 (TS2.18) biotin monoclonal antibodies followed by streptavidin-PE crosslinking. As shown, surface hCD2 expression is detected only in the transgenic mice and is restricted to the T-cell lineage. No significant differences in the percentages of DN, DP, CD4SP, and CD8SP subpopulations were detected between transgenic and nontransgenic littermates.

endogenous TCR/CD3 complex. Comparison of the ability of the two CIDs to induce tyrosine phosphorylation of the TCRζ chimera demonstrated that AP1510 is more effective than FK1012-H2, similar to what was observed after transient transfection of the chimeric receptor into Jurkat T cells (not shown). Overall, these data demonstrate that intracellular crosslinking of the chimeric receptor leads to receptor tyrosine phosphorylation.

To determine if the TCRζ chimeric receptor could transduce signals further downstream, we examined whether phosphorylation of the chimeric receptor in primary thymocytes or T cells lead to the recruitment and activation of Zap70. For this experiment, cells were stimulated with CID, lysed, and immunoprecipitated with an antibody to Zap70. Immunoprecipitates were then examined for tyrosine phosphorylation of Zap70, as well as coimmunoprecipitation of the chimeric receptor. In contrast to what we observe in Jurkat cell experiments, recruitment and/or activation of Zap70 was not detectable after stimulation of the chimeric TCRζ protein in primary cells (data not shown). Nonetheless, it remained possible that functional signals were being transduced through the chimeric TCRζ chain, even though downstream activation events could not be detected at the biochemical level.

As a further test of this possibility, we investigated whether intracellular crosslinking of the chimeric TCRζ receptor could generate a calcium mobilization response. For these experiments, thymocytes or lymph node cells were loaded with the calcium indicator dyes, Fura Red and Fluo-3, and cells were stimulated with CID, anti-hCD2 antibody, or a combination of anti-CD3 and anti-CD4 antibodies. Figure 6 shows a representative calcium response of transgenic thymocytes (Fig. 6A) or lymph node T cells (Fig. 6B) to anti-CD3 plus anti-CD4 antibody crosslinking. FK1012-H2 at 1 μM was not able to induce calcium mobilization in the primary T cells or thymocytes (not shown). However, calcium mobilization was observed in similar experiments using the AP1510 dimerizer in transgenic thymocytes. Interestingly, this response had delayed kinetics relative to the response to anti-CD3 plus anti-CD4 crosslinking, starting at approximately 4 min after stimulation (Fig. 6A). Extracellular crosslinking of the chimeric receptor with anti-hCD2 antibody also induced a weak calcium mobilization response, but in

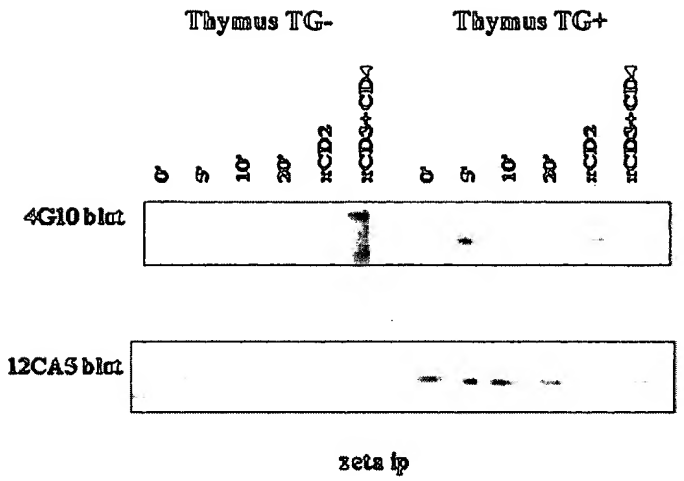

FIG. 5. CID stimulation of transgenic T cells induces tyrosine phosphorylation of the TCRζ chimera. Ten million thymocytes obtained from transgenic or nontransgenic littermates were stimulated with 1 μM AP1510 (1–20 min) or with anti-hCD2-biotin or anti-CD3 + CD4 biotin followed by streptavidin crosslinking (3 min) and lysates were immunoprecipitated with anti-TCRζ antibody (Santa Cruz). Immunoprecipitated proteins were resolved in a denaturing 10% SDS–PAGE gel. The Western blot was probed with antiphosphotyrosine antibody 4G10 (top panel) and blot was stripped and reprobed with anti-HA antibody 12CA5 (bottom panel). Stimulation with AP1510 and anti-hCD2 treatment induced specific tyrosine phosphorylation of the CD2ζFKBP-12 ×3 chimera, but no phosphorylation is obtained after crosslinking of the endogenous TCR/CD3 complex with anti-CD3 + anti-CD4 treatment.

this case, with similar kinetics to the anti-CD3 plus anti-CD4 antibody crosslinking controls (Fig. 6A). In contrast to the response in thymocytes, AP1510 stimulation did not induce calcium mobilization in transgenic peripheral T cells (Fig. 6B), perhaps due to different thresholds for activation in mature T cells. This latter possibility is consistent with the idea that thymocytes are required to recognize low-affinity TCR interactions during the positive selection process.

Activation of the TCRζ Chimeric Receptor Does Not Induce a Functional Response in Primary T Lymphocytes

To determine whether the signaling initiated by the chimeric TCRζ receptor resulted in physiologically relevant outcomes, we performed functional assays on transgenic thymocytes and peripheral T cells. As a first approach, we examined proliferative responses of thymocytes, lymph node cells, or splenocytes from transgenic mice after stimulation with CID. Figure 7 shows the results from a representative experiment. No proliferation was observed when transgenic cells were stimulated for three days with 0.1, 1, or 10 μM FK1012-H2 or AP1510 (Fig. 7A). In contrast, positive controls using anti-CD3 antibody, or anti-CD3 plus anti-CD28 antibodies, resulted in a strong proliferative response from both transgenic and nontransgenic T cells or thymocytes.

We also examined the possibility that intracellular crosslinking of the TCRζ chimera requires additional costimulatory signals to promote T-cell proliferation. To test this, parallel cultures were stimulated with the CID AP1510 together with anti-CD28 antibody, anti-CD4 antibody, or anti-CD2 plus anti-CD4 antibodies or in the presence of exogenous IL-2. None of these combinations of signals was sufficient to induce proliferation of primary transgenic thymocytes, lymph node cells, or splenocytes (data not shown). These results indicate that crosslinking the TCRζ chimera is unable to generate the signals necessary to induce T-cell proliferation. These data are consistent with a previous report using mice expressing an Fv-ζ chain chimeric protein (20). However, in this latter study, the authors were able to induce a proliferative response after stimulation of the chimeric receptor on primed, or previously activated, T cells. We tested this possibility in our system, using the same prestimulation protocol but we still detected no T-cell proliferation in response to CIDs (Fig. 7B).

As a second means of examining the functional responses of transgenic T cells to crosslinking of the chimeric TCR ζ chain, we investigated whether CIDs could induce the upregulation of activation markers. For these experiments, lymph node cells were stimulated with FK1012-H2 or AP1510 (0.1 and 1 μM) or with anti-hCD2 crosslinking. None of these treatments did induce upregulation of CD25, CD44, or CD69 (data not shown), while lymph node cells incubated with CD3+CD28 plate-bound antibody showed upregulation of these surface markers as well as a significant increase in cell size.

Since we observed no positive activation response after treatment of transgenic T cells or thymocytes with CIDs, we investigated whether the CIDs might be inducing an inhibitory signal leading to cell death. To assess cell death, a combination of AnnexinV and propidium iodide (PI) was used. However, treatment with CIDs induced no marked increase in apoptotic or dead cells expressing the chimeric receptor (data not shown).

Individual Crosslinking of the TCRζ Chain Induces No Downregulation of Coreceptors in Transgenic Thymocytes

As described above, conditional activation of the chimeric TCRζ chain protein in transgenic thymocytes generates a moderate calcium mobilization response after 4–5 min of stimulation. To investigate whether this level of signaling is sufficient to promote T-cell differentiation or thymic selection, we utilized two different *in vitro* assays. The first assay, referred to as a "dulling assay," has been used to examine TCR signaling leading to negative selection. This assay takes advantage of the fact that deletion of CD4+8+ thymocytes during negative selection is preceded by downregulation of the CD4 and CD8 coreceptors (46). For

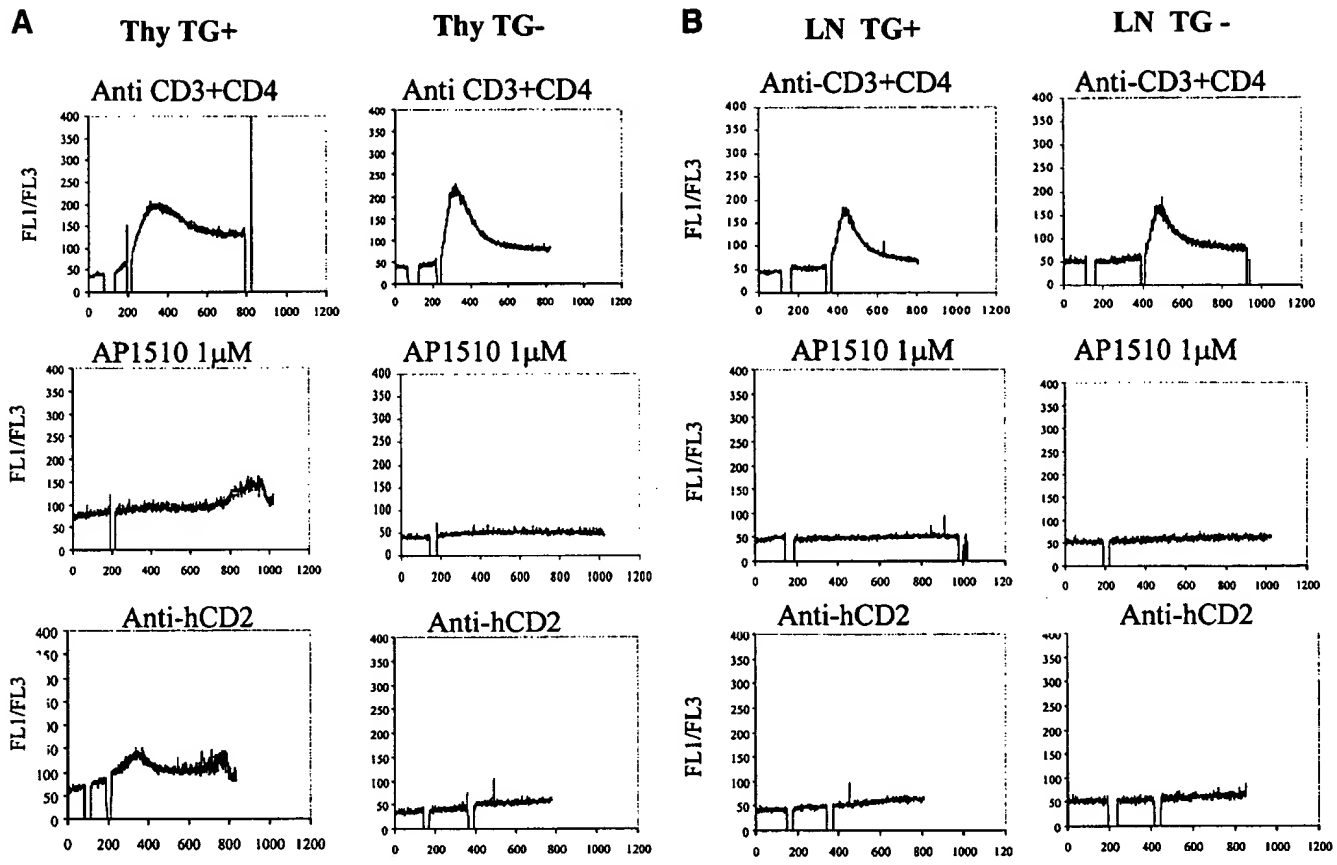

FIG. 6. Crosslinking of the CD2ζFKBP-12 ×3 chimera in transgenic thymocytes induces a partial calcium flux response. Thymocytes (A) and lymph node cells (B) from TCRζ transgenic mice and nontransgenic littermates stimulated with the AP1510 (1 μM). The upper plots show calcium mobilization using anti-CD3 + CD4-biotinylated antibodies followed by streptavidin crosslinking that were used as positive controls. CD3 + CD4 crosslinking resulted in a strong calcium mobilization in both transgenic and nontransgenic mice, while stimulation of these same cells with AP1510 and hCD2 crosslinking induced a slow and transient calcium mobilization in transgenic thymocytes but not in peripheral T lymphocytes. Ionomycin (500 ng/ml) was added as a control at 800 s to assess the efficiency of dye loading.

this assay, thymocytes were incubated for 24 h in the presence of CIDs or crosslinking antibodies followed by flow cytometry analysis. These experiments indicated that stimulation with CIDs was unable to induce specific downregulation of CD4/CD8 coreceptors on transgenic thymocytes (Fig. 8, left panels). Interestingly, despite the lack of CD4/CD8 downregulation, crosslinking of the chimeric TCRζ chain receptor by the CID did lead to the down regulation of this receptor from the thymocyte cell surface. This finding indicates that the interaction of the chimeric receptor with the CIDs does generate a signal sufficient for mediating receptor internalization (right panels).

Individual Crosslinking of the TCRζ Chain Promotes No T-Cell Development in Vitro

As a second assay for thymocyte selection, we performed fetal thymic organ cultures. Fetal day-16 and day-17 thymi were isolated and cultured in medium in the presence or absence of CIDs. *In vitro* T-cell development was assessed after 6 days of culture. Flow cytometry analysis of freshly isolated transgenic fetal day-16 thymocytes showed that expression of the transgenic TCRζ chimeric receptor was detectable at this developmental stage (not shown). Nonetheless, incubation with CIDs did not lead to significant differences in the generation of CD4 or CD8 single positive (SP) thymocytes *in vitro*.

One concern with the fetal thymic organ culture experiments described above is the fact that thymocytes from the transgenic mice already express endogenous TCR/CD3 complexes; thus, T-cell development progresses normally with no additions to the fetal thymic organ culture medium. Consequently, this initial assay would only detect perturbations of the normal developmental pattern in response to signaling through the chimeric receptor. To generate a more sensitive assay in which the chimeric TCRζ chain receptor might mediate developmental signals, CD2ζFKBP ×3 transgenic mice were crossed to either TCRζ/η-deficient or Rag-deficient mice. In TCRζ/η-deficient mice, T-cell development is blocked by the inability of the αβ-TCR to assemble, and thymocytes are predominantly arrested at the DP stage. In contrast, Rag-1-deficient thymocytes exhibit an earlier developmental block at the CD4-8 stage due to a deficiency in pre-TCR

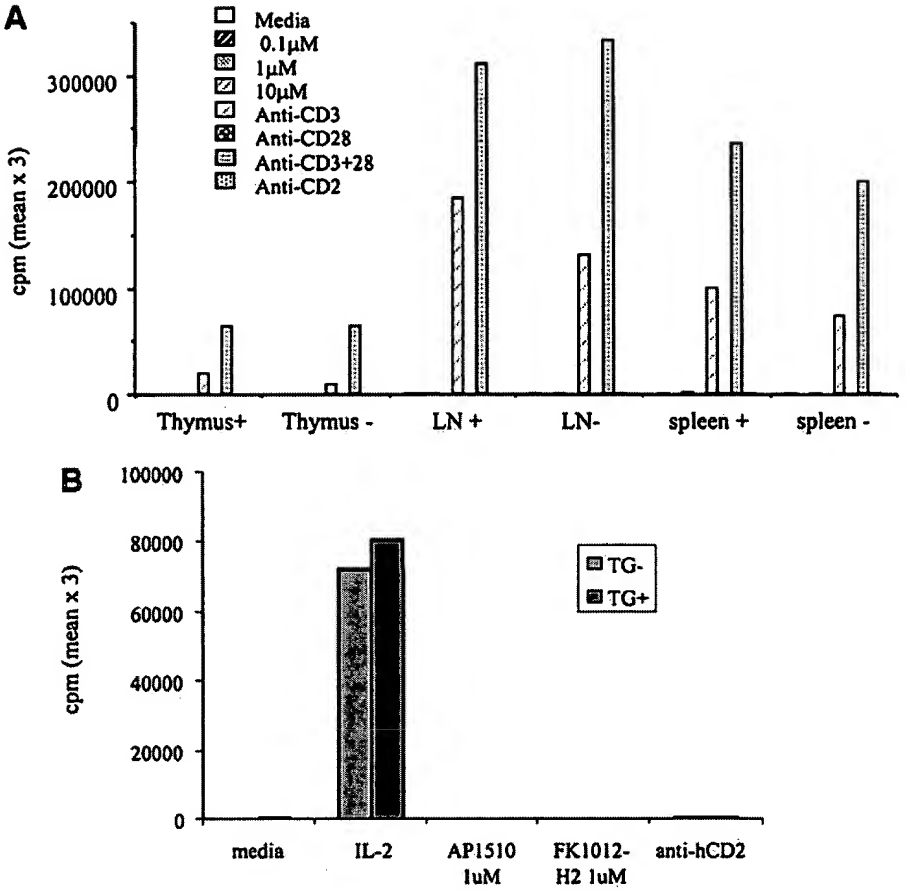

FIG. 7. Proliferative response of thymocytes, lymph node cells, and splenocytes from a transgenic mice (line 26) and nontransgenic littermates. (A) Thymocytes and lymph node cells were stimulated with the dimerizer AP1510 (0.1, 1, and 10 μM) in the presence of irradiated (3000 rads) syngeneic splenocytes. As controls, responses to plate-bound CD3, CD28, and CD3+CD28 or media alone were also performed. [^{3}H]Thymidine incorporation is represented as counts per minute and plotted on the ordinate. Values represent the mean of triplicate wells. (B) Proliferative response of prestimulated T lymphocytes after stimulation with CID or antibody crosslinking. For this experiment, T lymphocytes were preincubated for 48 h with anti-CD3 crosslinking and incubated for another 24 h in the presence of IL-2 (see Materials and Methods). T Lymphocytes were then stimulated with CIDs or anti-CD2 crosslinking. As a control, parallel cultures were incubated in IL-2 (50 U/ml). Bars represent mean values of triplicate cultures.

expression and signaling (47). Fetal thymic organ cultures were prepared from the TCRζ/η-deficient × CD2ζFKBP ×3 transgenic or Rag1-deficient × CD2ζFKBP ×3 transgenic mice. Even in these more sensitive systems, addition of CIDs could not promote T-cell maturation at either of the TCR-dependent checkpoints (Figs. 9A and 9B). These results indicate that the signal generated by intracellular crosslinking of the TCRζ chimeric receptor was not sufficient to substitute for either pre-TCR signaling in Rag-deficient mice or for mature $\alpha\beta$TCR signaling in TCRζ/η-deficient mice.

DISCUSSION

This study describes the role of signaling through the TCRζ chain in isolation from the other TCR/CD3 complex signaling proteins. In particular, we focused on the functional consequences of signaling through TCRζ in primary thymocytes and peripheral T cells. While this issue has been addressed in previous studies (17, 19, 20), our system utilizes a unique approach to activate signaling. Instead of using antibodies directed against extracellular domains of chimeric receptors to mediate crosslinking, our chimeric receptor is oligomerized by addition of an intracellular chemical inducer of dimerization. This system has the advantage of providing a more easily titrated means of inducing signaling and, further, is more likely to mimic the kinetics of receptor activation when TCR/CD3 complexes are engaged by MHC/peptide ligands on antigen presenting cells. Furthermore, this system has been proven successful in the study of signaling through many different receptors both *in vitro* and *in vivo*. For instance, in transgenic mice expressing a chimeric molecule consisting on the Fas antigen linked to one copy of FKBP-12, dimerization was sufficient to generate the signals required to induce cell death (apoptosis) of CD4+8+ thymocytes both *in vivo* and *in vitro* (35).

Although we detected no functional responses upon stimulation of the chimeric TCRζ receptor in primary T

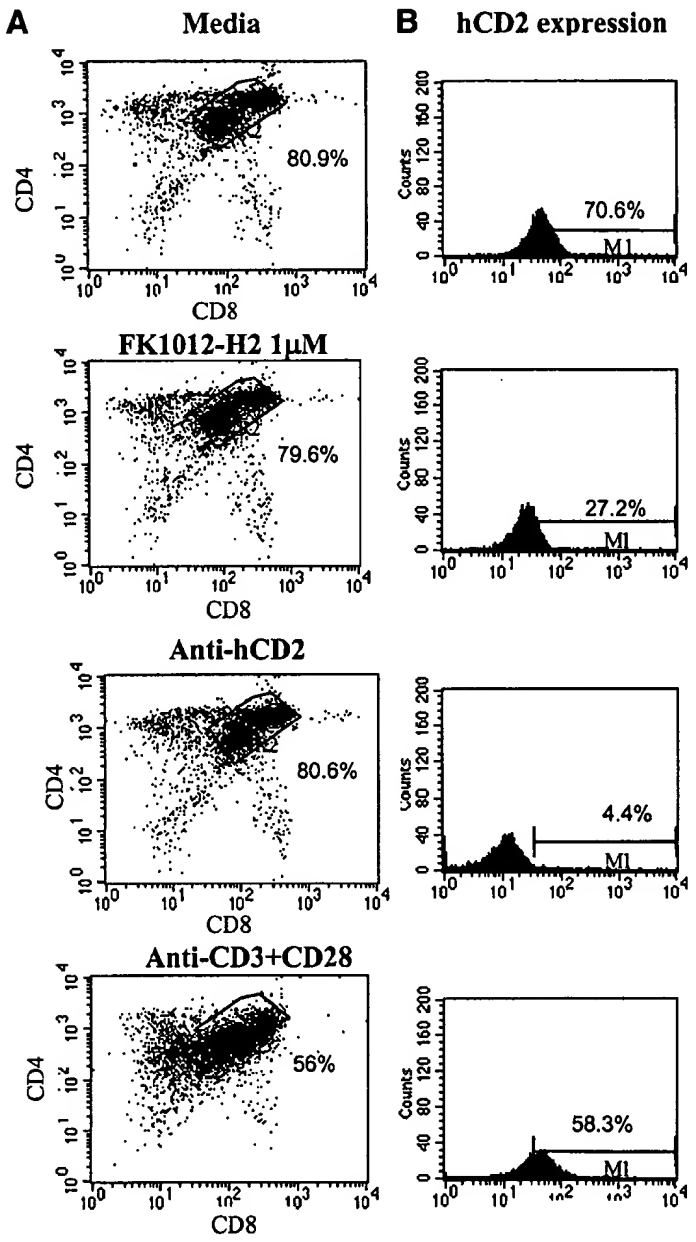

FIG. 8. Individual crosslinking of TCRζ chimera does not induce downregulation of CD4/CD8 coreceptors in TCRζ chimeric mice. Thymocyte dulling assay was performed by flow cytometric analysis of CD4 and CD8 coreceptor expression in thymocytes stimulated overnight with CIDs (1 μM) or with CD3+/− CD28 anti-hCD2 crosslinking. Primary antibodies were biotinylated and crosslinked with streptavidin (see Materials and Methods). (A) FACS dot blots showing the percentage thymocytes remaining in DP^{high} gated subpopulation. CD3+CD28 crosslinking induces significant coreceptor downregulation, which is not observed after hCD2 crosslinking or CID stimulation. (B) Single-parameter histograms of the hCD2 profiles from the DP^{high} gated population from representative examples of the indicated mice. Note that hCD2 expression is completely blocked in thymocytes from cultures stimulated with anti-hCD2 crosslinking and significant hCD2 downregulation is observed in the CID-stimulated transgenic thymocytes.

cells, we are confident that this receptor is competent for signaling. Data supporting this claim were obtained with Jurkat cells, as well as primary thymocytes and T cells from the transgenic mice expressing the receptor. For instance, in both Jurkat cells and primary cells, the chimeric TCRζ receptor became strongly phosphorylated as early as 1 min after stimulation with two different dimerizers, FK1012-H2 and AP1510. In addition, we detected a weak calcium mobilization response after stimulation of transgenic thymocytes with AP1510. As previously reported (48), TCRζ chain chimeric proteins expressed in Jurkat cells are capable of transducing signals leading to Zap70 recruitment and NFAT activation, and our studies were consistent with these findings, indicating the CD2-ζ-FKBP ×3 chimeric construct was fully functional. Therefore, our

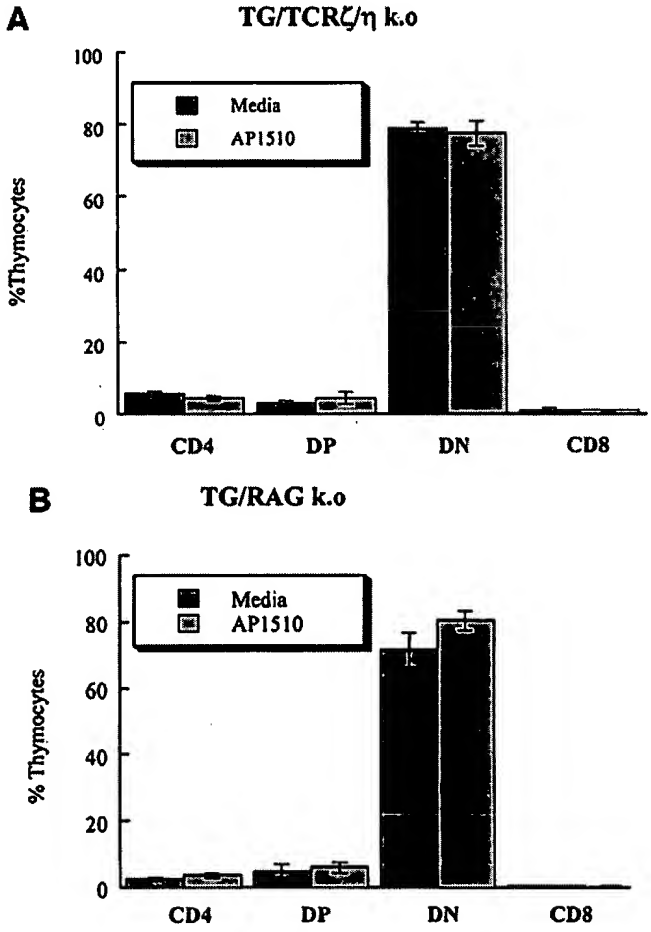

FIG. 9. Individual TCRζ chain crosslinking does not rescue the developmental block observed in Rag-1 and TCRζ/η knockout mice. This figure shows fetal thymic organ cultures from transgenic and nontransgenic mice bred in the Rag-1 and TCRζ/η knockout backgrounds. Newborn fetuses were used for these experiments, and individual thymic lobes were placed on to sponges soaked with either complete media or 1 μM AP1510 containing media. Media was changed every other day and cultures were analyzed after 6 days in culture. Thymocytes were then stained with anti-CD4-cychrome and CD8-FITC antibodies and percentages of DN, DP, and CD4SP, and CD8SP were calculated. (A) FTOC of transgenic thymocytes from mice bred on the ζ/η knockout background. Data represent the percentage of each thymic subpopulation in the final cultures and are represented as mean values of triplicate cultures. Standard errors obtained from these values are plotted and represented as Y bars in the histograms. (B) FTOC of transgenic and nontransgenic thymi bred on the RAG-1 KO background.

failure to induce primary T-cell activation or thymocyte differentiation upon stimulation of the chimeric TCRζ receptor is most likely due to the inability of signals through TCRζ alone to mediate these events *in vivo*.

There are two possible explanations for our failure to induce functional responses in primary cells by activating TCRζ alone. The first possibility is that the degree of oligomerization mediated by the CIDs was insufficient to generate a functional downstream signal. In support of this possibility, we found that the CID-induced phosphorylation of our chimeric TCRζ receptor was insufficient to recruit detectable levels of phosphorylated Zap70. This may have occurred due to incomplete phosphorylation of the TCRζ ITAMs. For instance, it has recently been shown that T-cell activation depends on the phosphorylation of all six tyrosines in the three TCRζ ITAM motifs, thereby leading to the association of several Zap70 molecules and favoring Zap70 hyperphosphorylation and activation (49). Another study also demonstrated that weak TCR engagement could generate early responses, such as calcium mobilization and acid release, in the absence of IL-2 production and T-cell proliferation (50). Finally, Kersh *et al.* (25) showed that partial phosphorylation of TCRζ ITAMs can itself generate a negative signal preventing T-cell activation, as is the case with some TCR antagonist peptide ligands. Thus, the extent of TCRζ phosphorylation seems to be a key factor in regulating different biological T-cell responses.

A second possible explanation for our findings is that signaling through the TCRζ chain alone in primary thymocytes or T cells is insufficient to mediate physiological responses. This may result from inefficient recruitment of the chimeric receptor to the detergent insoluble membrane fraction (lipid rafts), where phosphorylated Zap70 and many other signaling molecules are recruited upon TCR stimulation (51, 52). This may lead to the uncoupling of the initial TCRζ phosphorylation process from downstream signaling pathways, thereby preventing full T-cell activation. Recent studies have shown that the CD3δ chain component of the TCR/CD3 complex plays a critical role in the recruitment of the receptor complex to the appropriate membrane compartment and, further, that this localization is essential for signaling (53, 54). These data lend support to the notion that TCRζ alone may not properly localize to transduce downstream signals. An additional issue may also be the absence of CD3ε, which has been shown to recruit the important regulator, PI 3-kinase, via its phosphorylated ITAM (55). Lack of recruitment of PI 3-kinase might also prevent T-cell activation, as PI 3-kinase has been implicated in the activation of pathways involved in T-cell proliferation, apoptosis, and cytoskeletal reorganization (reviewed in 56).

Our results have shown that there are differences in the signaling capacity of the TCRζ chimera when comparing Jurkat T cells and primary transgenic thymocytes or mature T lymphocytes. One possible explanation that may account for these differences might be the variations in the lipid raft composition, which has been shown to differ between different cell lines or from cells at different developmental stages (57). In this context different amounts of TCRζ/CD3 components basally associated to cytoskeleton, levels of other raft-associated signaling molecules such as Lck, LAT and coreceptors, or changes in the distribution of cytoskeleton may result in different thresholds for T-cell activation.

One interesting issue is why our findings differ from those reported by others analyzing transgenic mice expressing TCRζ-chimeric receptors. One obvious possibility is the precise structure of the chimeric receptors generated. For instance, Brocker et al. (20) used a human CD3ε-specific Fv fragment and the complete TCRζ transmembrane and cytoplasmic sequence. With this receptor, the authors demonstrated that naïve T cells could not be induced to proliferate by extracellular crosslinking of the chimera, unless the cells had previously been stimulated. In contrast, Geiger and coworkers (19) used the extracellular and transmembrane domains of the MHC class II molecule, I-A^s. With this chimeric receptor, extracellular crosslinking was able to activate both naïve and memory T cells and, further, could promote their differentiation into CTLs, Th1, and Th2 effector cells. However, recent reports by T. Brocker (58) and Geiger and collaborators (59), using chimeric receptors containing the TCRζ cytoplasmic tail fused to an Fv fragment or to the extracellular and transmembrane domain of the H-$2K^b$ molecule, have provided further evidence that TCRζ alone cannot induce an effective T-cell activation response in primary T lymphocytes (cytokine production and effector function), implying that other signaling molecules such as TCR/CD3 components and/or coreceptors or costimulators may be required. In another study, Shinkai *et al.* (17) used the cytoplasmic tail of TCRζ or CD3ε chains fused to the extracellular and transmembrane domain of the human IL-2 receptor. This latter study concluded that both TCRζ and CD3ε cytoplasmic tails could generate similar signals for T-cell activation and T-cell development. However, in this system, Con A supernatants were used in the proliferation assays, and the authors did not exclude the role of endogenous CD3ε or TCRζ chains in the functional responses observed.

The ability of some chimeric TCRζ receptors to generate functional responses may depend on the level of expression of the chimeric receptor as well as the extent of receptor crosslinking induced. Our transgene was expressed under the hCD2 promoter and resulted in significant expression of the chimeric receptor similar to that obtained in a previous report using the same promoter (19). Our system, with its unique intracellular oligomerization process, may very well mediate the least robust form of receptor signaling. None-

theless, we would argue that this approach mimics *bona fide* TCR engagement more closely than does total cell surface receptor crosslinking using antibodies to extracellular domains.

On the other hand, particular transmembrane and/or extracellular domains may either facilitate or exclude the chimeric receptor from recruitment to the lipid rafts (60). The characteristics of transmembrane domains that confer translocation competence to the lipid rafts are not known. Our chimeric receptor bears the extracellular and transmembrane domains of the human CD2 molecule, which in principle, should not get excluded from recruitment to lipids rafts. However, stable residency of the receptors in the rafts after crosslinking may require initiation of signaling and interaction with the actin cytoskeleton (60), which would favor the formation of the immunological synapse leading to full T-cell activation.

In conclusion, our transgenic mice model shows that signaling through TCRζ alone is unable to generate the complete set of signals required for T-cell activation and development, suggesting that additional TCR/CD3 components or coreceptors may be required to achieve a functional response. With the experimental system we have established, we can sequentially add additional components by generating double or triple transgenic mice in which CIDs can be used to heterodimerize TCRζ with other CD3 components and/or coreceptors. This system will allow us to investigate the minimal receptor components needed for each T-cell response. Furthermore, different doses of dimerizer can be used to induce different degrees of oligomerization and thus to generate signals of varying intensity. In this way, we hope to evaluate the role(s) of individual TCR/CD3 signaling components *in vivo*.

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